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https://academic.oup.com/molehr/article/22/10/681/2236541,
https://doi.org/10.1093/molehr/gaw002

Cell fate in animal and human blastocysts and the determination of viability.

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Abstract

Understanding the mechanisms underlying the first cell differentiation events in human preimplantation development is fundamental for defining the optimal conditions for IVF techniques and selecting the most viable embryos for further development. However, our comprehension of the very early events in development is still very limited. Moreover, our knowledge on early lineage specification comes primarily from studying the mouse model. It is important to recognize that although mammalian embryos share similar morphological landmarks, the timing and molecular control of developmental events may vary substantially between species. Mammalian blastocysts comprise three cell types that arise through two sequential rounds of binary cell fate decisions. During the first decision, cells located on the outside of the developing embryo form a precursor lineage for the embryonic part of the placenta: the trophectoderm and cells positioned inside the embryo become the inner cell mass (ICM). Subsequently, ICM cells differentiate into embryonic lineages that give rise to a variety of tissues in the developing foetus: either the epiblast or extraembryonic primitive endoderm. Successful formation of all three lineages is a prerequisite for implantation and development to term. A comprehensive understanding of the lineage specification processes in mammals is therefore necessary to shed light on the causes of early miscarriages and early pregnancy pathologies in humans.

Keywords: mouse embryo, human embryo, lineage specification, mammalian embryo development, IVF

Introduction

IVF has become an everyday reality in modern society. However, its efficiency still remains unsatisfactory, forcing many couples to undergo multiple IVF cycles at significant extra cost (CDC, 2012; HHS, 2014). One of the major challenges faced by doctors performing IVF is the assessment of viability and quality of human embryos allocated for uterine transfer (Jones *et al*., 2008). Despite significant progress in recent years, the current methods of assessing embryo viability provide only a rough guide, rather than a conclusive prediction of the developmental potential of individual embryos. The crucial limiting factor is our lack of a comprehensive understanding of the key factors that can influence preimplantation development and what constitutes a healthy human embryo.

Our knowledge of mammalian development comes primarily from studying the mouse model. Information about human embryos is still sparse and fragmentary. Mammalian development significantly differs from the development of many other model organisms, as maternal determinants do not seem to play a role in lineage specification. Initially in the developing mammalian embryo, all cells have the same developmental potential and are 'totipotent'—defined as the ability to contribute to all embryonic and extraembryonic lineages. During subsequent development, polarized cells on the outside of the embryo differentiate into the trophectoderm (TE) an extraembryonic lineage that later contributes to the placenta (Gardner *et al*., 1973; Copp, 1979; Papaioannou, 1982; Gardner, 1983; Nagy *et al*., 2003a). Non-polarized inside cells also lose their totipotency and form either epiblast (EPI) or primitive endoderm (PrE; hypoblast). PrE is an extraembryonic lineage that contributes to the yolk sac, whereas EPI cells give rise to all cells of the developing foetus but cannot contribute to TE or PrE, thus defining them as 'pluripotent' (Gardner and Rossant, 1979; Gardner, 1998; Kurimoto *et al*., 2006; Saiz and Plusa, 2013). Although mammalian embryos are highly regulative and can compensate for experimental disruptions to their structure and cell number, successful formation of all three lineages is a prerequisite for implantation and further development to term. Failure to establish the TE lineage leads to a failure in implantation (Chawengsaksophak *et al*., 2004; Strumpf *et al*., 2005) whereas problems in PrE formation can affect both the exchange of nutrients and metabolites with the maternal uterine environment and the establishment of axial patterning in the developing embryo (Soudais *et al*., 1995; Farese *et al*., 1996; Morrisey *et al*., 1998). Finally, without the EPI, the embryo proper cannot form (Niwa *et al*., 2000; Avilion *et al*., 2003; Mitsui *et al*., 2003). Identifying factors that contribute to early lineage specification in humans is crucial for improving procedures of assisted reproduction techniques (ART). Moreover, a deep understanding of the processes that govern early cell fate decisions and lineage specification in mammals is critical to unravelling the causes of early miscarriage and early pregnancy pathologies.

In this review, we provide insights into the cellular and molecular events that lead to the establishment of the three lineages of the blastocyst in the mouse and compare it with early development of other mammals, including human. We also discuss how knowledge of the mechanisms of cell fate specification during development can be used to refine embryo selection and IVF culture criteria

Lessons from mouse

Low maintenance costs, ready availability and a well-developed *in vitro* culture system, coupled with easy access to a wide variety of transgenic/reporter lines, make mouse embryos the most popular model for studying mammalian development. Thus, it is not surprising that the majority of data on early development, specification of early cell lineages and the mechanisms controlling pluripotency in mammalian embryos come from the mouse.

In the mouse, as in other Eutherians, the period of development between fertilization and implantation is called 'preimplantation development'. During this time, the 1-cell embryo (zygote) undergoes three rounds of cleavages to produce an embryo with eight similar-looking, totipotent cells called blastomeres that retain the ability to contribute to all embryonic and extraembryonic lineages (Tarkowski and Wroblewska, 1967; Kelly, 1977; Suwińska *et al*., 2008). At the 8-cell stage, the first major differentiation event takes place. Initially round blastomeres expand their cell–cell contact area in an E-cadherin-dependent process called compaction and form a ball-like structure called a morula (Johnson and Ziomek, 1981; Pratt *et al*., 1982; Larue *et al*., 1994; Fierro-Gonzalez *et al*., 2013), in which individual blastomeres cannot be easily distinguished (Fig. 1). These profound structural changes are related to reorganization of cytoskeletal elements, endosomes and microtubule-organizing centres within each blastomere (Johnson and McConnell, 2004). Concomitantly, blastomeres polarize and exhibit an outward-facing apical surface, marked by the presence of microvilli (Calarco and Epstein, 1973; Handyside, 1980; Reeve and Ziomek, 1981; Johnson and Ziomek, 1983), which assembles a protein complex comprising the partitioning defective (PAR) molecules PAR3/PAR6 and atypical protein kinase C (aPKC) (Pauken and Capco, 1999; Plusa *et al*., 2005; Vinot *et al*., 2005). The baso-lateral domain is marked by the presence of PAR1 and the E-CADHERIN/B-CATENIN complex (Calarco and Epstein, 1973; Reeve and Ziomek, 1981; Vestweber *et al*., 1987; Guo and Kemphues, 1996; Louvet *et al*., 1996; Pauken and Capco, 1999, 2000; Vinot *et al*., 2005). Stable apical and basal domains are established and maintained thanks to the formation of adherens junctions and by the later development of tight junctions and desmosomes. Organization of tight junctions is also a prerequisite for the process of cavitation, which leads to formation of the next developmental stage: the blastocyst (Calarco and Brown, 1969; Nadijcka and Hillman, 1974; Ducibella *et al*., 1975; Fleming *et al*., 1989; Fleming and Hay, 1991; Thomas, 2004). By the time the morula reaches the 16-cell stage it is multi-layered, with outer cells maintaining apical-basal polarity and apolar inner cells. The outer layer of cells later forms the first epithelium, the TE (Tarkowski and Wroblewska, 1967), whilst the cells positioned inside become the ICM of the embryo. The development of cellular polarity is necessary for correct lineage specification, as interfering with the function of polarity proteins affects blastocyst morphogenesis and TE:ICM lineage allocation (Thomas, 2004; Plusa *et al*., 2005; Vinot *et al*., 2005; Alarcon, 2010). The process of compaction and polarization triggers a series of events during which inside cells transition from totipotent to pluripotent and outside cells commit to TE, losing the ability to contribute to other embryonic lineages between the 32- and 64-cell stages (Handyside, 1978; Szczepanska *et al*., 2011).

At around the 30-cell stage, a process of cavitation leads to the formation of a blastocyst (Smith and McLaren, 1977), with TE encapsulating both ICM and a fluid-filled cavity. During this process, the inner cells are pushed towards one side of the embryo, and the first axis, the embryonic–abembryonic axis is formed. After blastocyst formation, some of the ICM cells differentiate into the PrE (called hypoblast in other species), which forms part of the inner lining of the blastocyst cavity. The cells surrounded by both TE and PrE form EPI—the origin of the embryo proper and a source of embryonic stem cells (Brook and Gardner, 1997; Dard *et al*., 2008; Saiz *et al*., 2015; rev. Saiz and Plusa, 2013).

The importance of both extraembryonic lineages is paramount. TE is responsible for implantation and contributes to the placenta. PrE develops into parietal and visceral endoderm, which forms the majority of the yolk sac (Gardner and Johnson, 1972; Gardner *et al*., 1973; Copp, 1979; Gardner and Rossant, 1979; Papaioannou, 1982; Gardner, 1983; Nagy *et al*., 2003b) and it also contributes to the definitive endoderm (Kwon *et al*., 2008). Furthermore, PrE plays a pivotal signalling role in the formation of the antero-posterior axis during gastrulation (Beddington and Robertson, 1999; Nagy *et al*., 2003b; Stern and Downs, 2012).

Two major signalling pathways—*H*ippo and *F*ibroblast *G*rowth *F*actor/*E*xtracellular signal-*R*egulated *K*inase—direct differentiation and segregation of extraembryonic lineages as well as the allocation of a pluripotent group of cells that form the EPI (Chazaud *et al*., 2006; Nishioka *et al*., 2008; Lanner and Rossant, 2010; Artus and Chazaud, 2014). The aforementioned differences in cell position and cell polarity after compaction lead to differential activation of the Hippo pathway between inside and outside blastomeres (Nishioka *et al*., 2008; Sasaki, 2010). The Hippo pathway remains inactive in outer cells, thus allowing translocation of the transcriptional cofactor, Yes-associated protein (YAP) and its closely related paralogue, WW-domain containing transcriptional regulator WWTR1 into the nucleus. YAP/WWTR1 binds the transcription factor, TEA-domain protein 4 (TEAD4), which activates expression of TE-specific genes responsible for maintenance of the lineage, such as a caudal-related homeodomain transcription factor (*Cdx2*) and GATA-binding protein 3 (*Gata3*) (Chawengsaksophak *et al*., 2004; Strumpf *et al*., 2005; Yagi *et al*., 2007; Ralston and Rossant, 2008; Home *et al*., 2009; Nishioka *et al*., 2009; Ralston *et al*., 2010). In inner cells on the other hand, the Hippo pathway is active, thus preventing YAP/WWTR1 from relocating to the nucleus. As a result, TE-specific genes are suppressed in these cells while the expression of pluripotency genes such as POU domain class 5 transcription factor 1/Octamer-binding transcription factor 4 (*Pou5f1/Oct4*), SRY-*Box2* (*Sox2*) and *Nanog* is maintained. In addition, CDX2-POU5F1/OCT4 antagonism has been suggested to contribute to TE versus ICM specification. According to this model, CDX2 suppresses *Pou5f1/Oct4* expression in future TE, whereas POU5F1/OCT4 may contribute indirectly to *Cdx2* repression in inside cells (Niwa *et al*., 2005; Dietrich and Hiiragi, 2007; Sasaki, 2010).

Lineage allocation within the ICM is influenced by fibroblast growth factor/mitogen-activated protein kinases (FGF/MAPK) signalling (Arman *et al*., 1998; Nichols *et al*., 2009; Yamanaka *et al*., 2010; Frankenberg *et al*., 2011; Kang *et al*., 2013; Krawchuk *et al*., 2013). It is widely accepted that in the mouse, the increased activity of FGF signalling in the developing blastocyst results in specification of two types of inner cells: the population expressing GATA-binding protein 6/4 (Gata6/Gata4) and SRY-Box17 (*Sox17*) responds to FGF signalling and contribute to a cavity-lining PrE layer. Cells that do not respond to FGF4 and maintain expression of pluripotency factors *Nanog*, *Sox2* and *Pou5f1/Oct4* (Morrisey *et al*., 1998; Nichols *et al*., 1998; Koutsourakis *et al*., 1999; Avilion *et al*., 2003; Chambers *et al*., 2003; Mitsui *et al*., 2003; Silva *et al*., 2009; Messerschmidt and Kemler, 2010; Niakan *et al*., 2010; Artus *et al*., 2011; Kang *et al*., 2013; Wicklow *et al*., 2014).

The cells that do not respond to FGF4 become naïve EPI, the innermost group of cells nestled between the TE and PrE, that give rise to all cell types of the adult body. Suppression of FGF signalling in mouse embryos results in a shift of ICM fate towards EPI with high *Nanog* expression, whilst the increase of FGF signalling results in the majority of ICM cells differentiating into PrE (Nichols *et al*., 2009; Yamanaka *et al*., 2010; Grabarek *et al*., 2012). As shown via analysis of mutants, the ligand FGF4, its receptor FGFR2 and the adaptor protein GRB2 (which mediates FGF/MAPK signalling) are all required for PrE formation (Feldman *et al*., 1995; Wilder *et al*., 1997; Arman *et al*., 1998; Cheng *et al*., 1998; Chazaud *et al*., 2006; Kang *et al*., 2013; Krawchuk *et al*., 2013).

From the morula to mid-blastocyst stage, various transcription factors with later lineage-specific expression are initially co-expressed in cells (Dietrich and Hiiragi, 2007; Plusa *et al*., 2008) (Fig. 2). Past the 64-cell stage, two cell populations can be distinguished within the ICM: one exclusively expressing markers of PrE, such as GATA4, GATA6, SOX7, SOX17 and platelet-derived growth factor receptor alpha (Pdgfra) and the other expressing markers of EPI, such as NANOG and SOX2 (Chazaud *et al*., 2006; Kurimoto *et al*., 2006; Plusa *et al*., 2008; Guo *et al*., 2010; Artus *et al*., 2011). Initially, these two ICM cell populations are positioned in a mosaic, apparently random manner described as 'salt-and-pepper' pattern (Chazaud *et al*., 2006). In embryos with more than ~100 cells they become segregated into their respective layers by mechanisms involving random cell movements, positional signals and selective apoptosis (Plusa *et al*., 2008; Meilhac *et al*., 2009; Yamanaka *et al*., 2010).

The process of mammalian preimplantation development is a paradigm of highly regulative self-organization rather than inherent pre-patterning (Fig. 2). Morphogenesis and lineage specification do not rely on maternal determinants, but instead are a result of intercellular interactions, spatial positioning and external signals from the embryo microenvironment (Grabarek *et al*., 2012; Saiz *et al*., 2015). Lineage specification in the early mouse embryo is driven by a combination of physical, chemical and temporal factors. This is probably true for all other mammals since they do not show clear evidence of asymmetrically distributed maternal determinants, despite other inter-species molecular differences.

Mouse and human

Direct observation of human preimplantation development is often difficult because of ethical issues, scarcity and inconsistent quality of available material in the form of *in vitro* produced and cultured embryos. However, a number of publications addressing human preimplantation development have been published in recent years.

In general, human and mouse preimplantation embryos share similar morphological landmarks, although timing of the events differs between the two species (Fig. 3). In human embryo, early cleavage divisions are followed by compaction at the morula stage and then cavitation leading to formation of a blastocyst at 4–5 days post coitum (dpc), which implants at 6–8 dpc (Hertig *et al*., 1959; Steptoe *et al*., 1971). As in the mouse, three cell lineages—EPI, hypoblast (PrE) and TE—are distinguishable by the late blastocyst stage (De Paepe *et al*., 2014). By and large, the same lineage-specific transcription factors are present in human and mouse embryos; however, the localization and timing of their expression differ (Kimber *et al*., 2008; Cauffman *et al*., 2009; Bernardo *et al*., 2011; Roode *et al*., 2012; Niakan and Eggan, 2013; Blakeley *et al*., 2015). Importantly, human and mouse embryos differ in their response to activation/inhibition of FGF signalling that was proposed to play crucial roles in EPI/PrE lineage specification in the mouse (Kuijk *et al*., 2012; Roode *et al*., 2012).

To date, little is known about the mechanism of TE specification in human. During human development, the first signs of compaction can be observed at 4 dpc (10 cells), when the microvilli start to exhibit a polarized distribution on the outer (free) surface of blastomeres (Nikas *et al*., 1996). This is coincident with the appearance of the gap junction protein connexin CX43 (Hardy *et al*., 1996) and E-CADHERIN localization to the cell–cell contact areas at 4 dpc (Alikani, 2005). The role of YAP in human embryonic development has not been studied (Kuijk *et al*., 2015); however, it was shown that YAP is activated during the process of human embryonic fibroblast reprogramming into induced pluripotent stem cells (iPSCs) (Lian *et al*., 2010), suggesting that Hippo signalling could be involved in the acquisition of human pluripotency *In vivo*.

One important difference between mouse and human development is localization of the key TE-associated transcription factor, CDX2, which in the mouse was proposed to be essential for TE identity and maintenance (Strumpf *et al*., 2005). In the mouse, CDX2 is observed for the first time at the morula stage, prior to TE formation (Dietrich and Hiiragi, 2007). In human embryos, CDX2 localization is also associated with the TE (Bernardo *et al*., 2011; Niakan and Eggan, 2013; Blakeley *et al*., 2015); however, it seems to be absent from the morula and early blastocyst stage, when TE can be morphologically distinguished. These observations support the notion that in human embryos CDX2 is not involved in TE specification (Chen *et al*., 2009; Niakan and Eggan, 2013). Interestingly, mouse embryos depleted of CDX2 can proceed to an equivalent early blastocyst stage, proving that other factors can drive TE specification in the absence of CDX2 (Ralston and Rossant, 2008).

Coincidentally, in human embryos the pluripotency-associated factor OCT4 was first detectable at the 8-cell stage and persisted in all cells including TE up to ~100 cell stage (Day 4), and became solely restricted to ICM by Day 6 (Chen *et al*., 2009; Roode *et al*., 2012; Niakan and Eggan, 2013). This clearly indicates that mutual CDX2-OCT4 antagonism is not necessary for ICM/TE segregation in human embryos (Chen *et al*., 2009; Niakan and Eggan, 2013).
Interestingly, it has been shown that TE cells of human blastocysts are able to develop into both ICM and TE, and therefore are not committed to only one lineage even at the blastocyst stage (De Paepe *et al*., 2013).

Recent work by Krivega *et al*. (2015) implicated a role for WNT3 and membrane-associated B-CATENIN in propagation of human TE. These data are in sharp contrast to mouse studies that demonstrate that PORCN-dependent embryonic WNT signals are not required for lineage specification in preimplantation development or for implantation itself, and identified gastrulation as the first PORCN/WNT-dependent event in mouse embryonic development (Biechele *et al*., 2013).

The transcription factors instrumental for EPI (NANOG, SOX2) and hypoblast (PrE: GATA6, GATA4, SOX17) formation in the mouse embryo are also present in the human blastocyst although the timing of their expression may differ from mouse embryos. SOX2 transcripts have been found as early as the 4-cell stage (Kimber *et al*., 2008); however, no clear nuclear localization of SOX2 was demonstrated at this early stage (Cauffman *et al*., 2009). The first evidence of nuclear localized SOX2 in human embryos was reported in the compacted morulae and the ICM of the early blastocyst, whereas the nuclear-localized NANOG protein was present only at later stages of blastocyst development (Cauffman *et al*., 2009).

Similar to the mouse, SOX17 is first detected at the early blastocyst stage (~32 cell) in human (Niakan and Eggan, 2013). At mid-blastocyst stage, GATA6 is localized broadly in human, similar to the mouse early blastocyst and overlapping with NANOG, which is found only in a subset of ICM cells at this stage (Roode *et al*., 2012). By the late blastocyst stage (7 dpc), overlapping expression of *GATA6, GATA4* and *SOX17* is localized in (putative) hypoblast cells starting to line the blastocoel cavity, and is exclusive from NANOG expression that is found in the putative EPI (Kimber *et al*., 2008; Cauffman *et al*., 2009; Roode *et al*., 2012). Therefore by the end of the preimplantation period, the localization of key lineage-specific transcription factors in the human embryo (7 dpc) resembles the implanting mouse embryo (4.5 dpc). Interestingly, despite some differences in timing of localization of the lineage-specific transcription factors in both species, we can observe a clear progression from an overlapping to mutually exclusive pattern of expression of the EPI and hypoblast (PrE) markers (Fig. 3).

In the differentiation of mouse PrE (hypoblast) and EPI, FGF signalling plays an instructive role (Yamanaka *et al*., 2010; Kang *et al*., 2013). FGFR2, although strongly expressed in mouse blastocyst stage embryos, was not present in human blastocyst at 6 dpc (Rappolee *et al*., 1998; Roode *et al*., 2012; Kunath *et al*., 2014). Consistent with this finding, the formation of human hypoblast does not depend on FGF signalling. Contrary to the mouse, in human preimplantation embryo inhibition of Mitogen/Extracellular signal-regulated Kinase (MEK) or FGF receptor does not interfere with hypoblast specification. Even with a lack of FGF signalling, the hypoblast lineage is still formed with the same efficiency as in unperturbed embryos, as shown by the presence of GATA6 and GATA4 factors, and formation of EPI or TE is also not affected (Greber *et al*., 2010; Kuijk *et al*., 2012; Roode *et al*., 2012).

Other mammalas

Although most of our knowledge on early mammalian development is based on studies in the mouse, there is a growing body of data concerning other mammalian species (Taft, 2008; rev. Kuijk *et al*., 2015). These data show some notable differences between species, although stage-equivalent comparisons can sometimes be difficult. This is due to the fact that in many mammalian species, the preimplantation period of development extends far beyond the hypoblast/EPI specification stage, while TE undergoes additional differentiation in preparation for attachment and implantation (Bazer *et al*., 2009; rev. Kuijk *et al*., 2015).

As in human, little is known about Hippo pathway involvement in TE versus ICM differentiation in other mammals. The TE-specific transcription factor, CDX2, is localized specifically in TE cells of bovine (Kuijk *et al*., 2012; Madeja *et al*., 2013), porcine (Kuijk *et al*., 2008) and rhesus macaque blastocysts (Harvey *et al*., 2009), and has been shown to affect proliferation of bovine TE cells in knockdown experiments (Berg *et al*., 2011). In agreement with the mouse studies, but in contrast to human studies, nuclear localization of CDX2 protein was also reported at the morula stage in Rhesus (Harvey *et al*., 2009), and bovine embryos (Madeja *et al*., 2013), confirming the mRNA expression data (Kuijk *et al*., 2008). POU5F1/OCT4 was detected in ICM and in TE of human, bovine and porcine blastocysts, but only in ICM of murine blastocysts (Kirchhof *et al*., 2000; Kuijk *et al*., 2008; Berg *et al*., 2011), and also in morulae of rhesus macaque (Harvey *et al*., 2009) and bovine (Madeja *et al*., 2013). This late expression of POU5F1/OCT4 is likely linked to maintenance of totipotency, as bovine TE cells have been shown to contribute to ICM in chimaeras (Berg *et al*., 2011). This property is lost at later stages, coinciding with a decrease in POU5F1/OCT4 levels in TE.

Disruption of WNT signalling in preimplantation ungulate embryos affects the ratio of TE versus ICM specification, although both lineages are specified and embryos reach the blastocyst stage (Berg *et al*., 2011). Activation of WNT signalling reduced the number of TE cells in both pig (Lim *et al*., 2013) and bovine (Denicol *et al*., 2013, 2014) embryos, coincident with activation of several pluripotency genes (Madeja *et al*., 2015), while inhibition of WNT resulted in an increased number of TE cells and promoted hatching (Lim *et al*., 2013; Denicol *et al*., 2014).

The second specification event in the preimplantation mammalian embryo—hypoblast versus EPI—in the mouse is largely linked to interplay between GATA6 and NANOG transcription factors, and dependent upon FGF signalling. Although NANOG has been shown to be one of the key factors associated with EPI and the pluripotent state, its expression pattern varies greatly between the species. In rhesus macaque, NANOG is present at the morula stage and later, in the ICM (Harvey *et al*., 2009). Interestingly, it has not been detected at equivalent stages in porcine embryos (Kuijk *et al*., 2008; Wolf *et al*., 2011; Cao *et al*., 2014), although it is present in the embryonic disc of post-hatching pig blastocysts (Wolf *et al*., 2011). In bovine embryos, NANOG was not detected at the morula stage in some of the studies, but its expression was specific to a subset of ICM cells, presumably EPI precursors, at the blastocyst stage (Kuijk *et al*., 2008, 2012). Others reported the presence of *NANOG* mRNA in bovine embryos beginning at the morula stage (Madeja *et al*., 2013).

GATA6 was detected in a subset of porcine and bovine ICM cells (Kuijk *et al*., 2008, 2012) and shown to become progressively mutually exclusive from a NANOG-positive population in bovine embryos, most likely marking the emerging hypoblast population. GATA6- and NANOG-positive bovine ICM cells were shown to later segregate into two compartments (Denicol *et al*., 2014) in a manner similar to human and mouse embryos (Plusa *et al*., 2008; Niakan and Eggan, 2013). GATA4, which in mouse and human localizes to a subset of GATA6-positive cells and marks the emerging hypoblast (PrE) population, was not detected in porcine embryos. In bovine embryos, GATA4 marks both ICM and TE, suggesting that in these species it is not a specific hypoblast marker (Kuijk *et al*., 2008, 2012).

In mouse, hypoblast versus EPI specification depends on FGF/MEK signalling (Arman *et al*., 1998; Nichols *et al*., 2009; Yamanaka *et al*., 2010; Frankenberg *et al*., 2011; Kang *et al*., 2013). Similar to the mouse, inhibition of MEK signalling in common marmoset embryos also leads to the ablation of PrE and an increase in numbers of EPI cells (Boroviak *et al*., 2015), while in bovine embryo culture the presence of exogenous FGF4 reduces the number of EPI cells (Kuijk *et al*., 2012). Depending on the time regime, this could lead to embryos forming that entirely lack a NANOG-positive population of cells. However, blocking the FGF receptor had no effect on embryonic development, while blocking the MEK signalling pathway showed only a slight bias towards EPI specification, with both EPI and hypoblast populations of cells still present (Kuijk *et al*., 2012). This suggests that the second lineage specification event in bovine embryos is linked to, but not dependent on, FGF signalling.

Can research on lineage segregation help increase the success of IVF techniques?

There are multiple elements that contribute to a successful pregnancy after IVF. Apart from the mother's health, embryo developmental potential is a major factor, which depends on successful formation of all three embryonic lineages. A lack of or reduced cell number in any of the first three cell lineages in mammalian blastocysts has striking effects on further development and often the affected embryos do not proceed beyond implantation (Feldman *et al*., 1995; Nichols *et al*., 1998; Mitsui *et al*., 2003; Chawengsaksophak *et al*., 2004; Ralston and Rossant, 2008; Kang *et al*., 2013; Schrode *et al*., 2014; Wicklow *et al*., 2014).

Identification of human embryos with the best developmental potential remains a big challenge in ART (Filho *et al*., 2010). Currently, there is no consensus about the most accurate method for assessing embryo quality but, in principle, all methods are based on some kind of morphological evaluations conducted in the IVF laboratory, including automatic (such as time lapse analysis) or semi-automatic analysis of microscope images (Filho *et al*., 2010; Montag *et al*., 2011). However, the formation of a morphologically normal-looking blastocyst does not necessarily infer correct lineage formation. Mutant mouse embryos that have not correctly specified one of their early cell lineages can be visually indistinguishable from their wild-type littermates until the blastocyst stage, yet their development does not progress beyond the peri-implantation period (Nichols *et al*., 1998; Mitsui *et al*., 2003; Chazaud *et al*., 2006; Nishioka *et al*., 2008; Kang *et al*., 2013; Schrode *et al*., 2014; Wicklow *et al*., 2014).

For obvious ethical and medical reasons, testing for correct lineage segregation in human embryos destined for transfer is not possible, thus it is difficult to ascertain how often failure in early cell lineage segregation contributes to pregnancy failure after embryo transfer. However, improved pregnancy success could be achieved by developing optimal embryo culture conditions in non-human animals that promote correct lineage specification and thus high quality embryos. Several reports show a significant effect of *in vitro* culture conditions on lineage allocation and lineage-specific gene expression in mammalian preimplantation embryos. For example, *in vitro* cultured rabbit embryos show elevated transcription levels of *NANOG* and *SOX2* (Henderson *et al*., 2014), as well as *POU5F1/OCT4* (Saenz-de-Juano *et al*., 2013) in comparison to *In vivo*-derived embryos. This effect can be rescued by regular medium renewal in culture (Saenz-de-Juano *et al*., 2013). Recent work on *in vitro*-derived equine embryos suggests that culture medium composition, and in particular glucose levels, can affect lineage allocation in the preimplantation embryo (Choi *et al*., 2015). So far, no comprehensive evaluation of the various culture regimes and their impact on correct lineage formation has been performed in the human embryo culture system. This is partially due to the fact that data on lineage specification in humans are still very limited and, thus far, no ‘gold standard’ of human preimplantation lineage development has been established.

As reported in 2009, over 40% of deliveries following IVF in the USA consist of twins or higher multiple births. This is due to the fact that in standard IVF cycles several embryos used to be transferred in order to increase the likelihood of pregnancy. Unfortunately, multiple pregnancies are often associated with an increased risk of preeclampsia, maternal haemorrhage, operative delivery, uterine rupture and pre-term labour (Crosignani and Rubin, 2000). Nowadays, these complications are usually avoided by transferring fewer (preferably only one) embryos to the mother's uterus during each of the IVF cycles (Filho *et al*., 2010; CDC, 2012; HHS, 2014). Therefore, it is of the utmost importance to create a system that will allow reliable assessment of embryo viability with a special emphasis on correct lineage formation. Various researchers address some of the aspects of this process, but further studies are needed in order to understand what constitutes a healthy human embryo. Since experimentation on human embryos is not possible for ethical reasons, the best possible understanding of early human embryology can only be achieved by also studying and extrapolating from the development of non-human mammalian species. However, as discussed in this review, concentrating solely on the mouse model is not sufficient, as many aspects of preimplantation development and lineage formation differ significantly between mouse and human. Finding a suitable mammalian model of human development remains a major challenge for the science of developmental biology.

Authors' roles

B.P. and A.P. provided the outline of the review. A.P., J.B.G., S.R.F. and B.P. wrote the manuscript. J.B.G. created the figures.

Funding

The work of AP is funded by National Science Centre grant number 2011/03/D/NZ3/03992. The work of the Plusa laboratory is funded by Leverhulme Trust project grant RG 73372. SRF work is supported by the University of Melbourne.

Conflict of interest

None declared.

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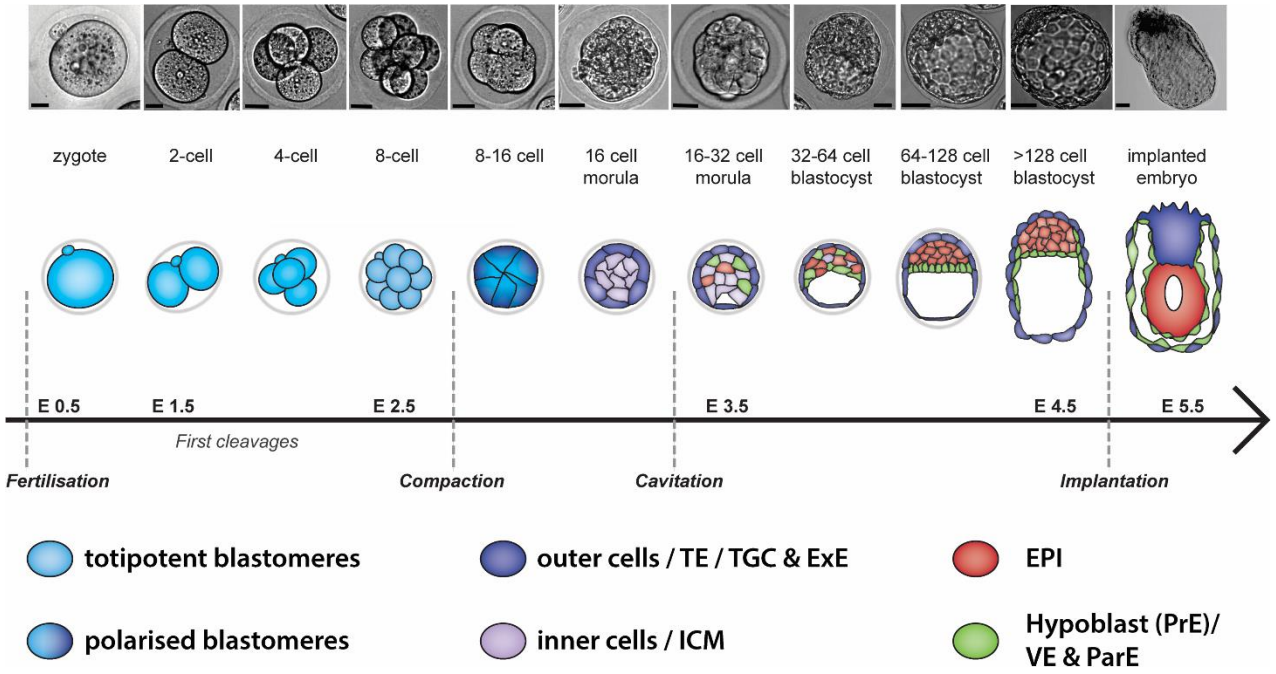

Figure 1: Early stages of mouse development. Schematic of the early stages of development, with corresponding names of the subsequent stages and the pictures of live embryos (top panel) above. The timeline below indicates the embryonic days (E). The main morphogenic events are indicated in italic and marked on the timeline. Colour legend describes respective embryonic cells and lineages. TE, trophectoderm; ICM, inner cell mass; EPI, epiblast; PrE, primitive endoderm; TGC, trophoblast giant cells; ExE, extraembryonic ectoderm; VE, visceral endoderm; ParE, parietal endoderm. Scale bars in the top panel are 20 µM.

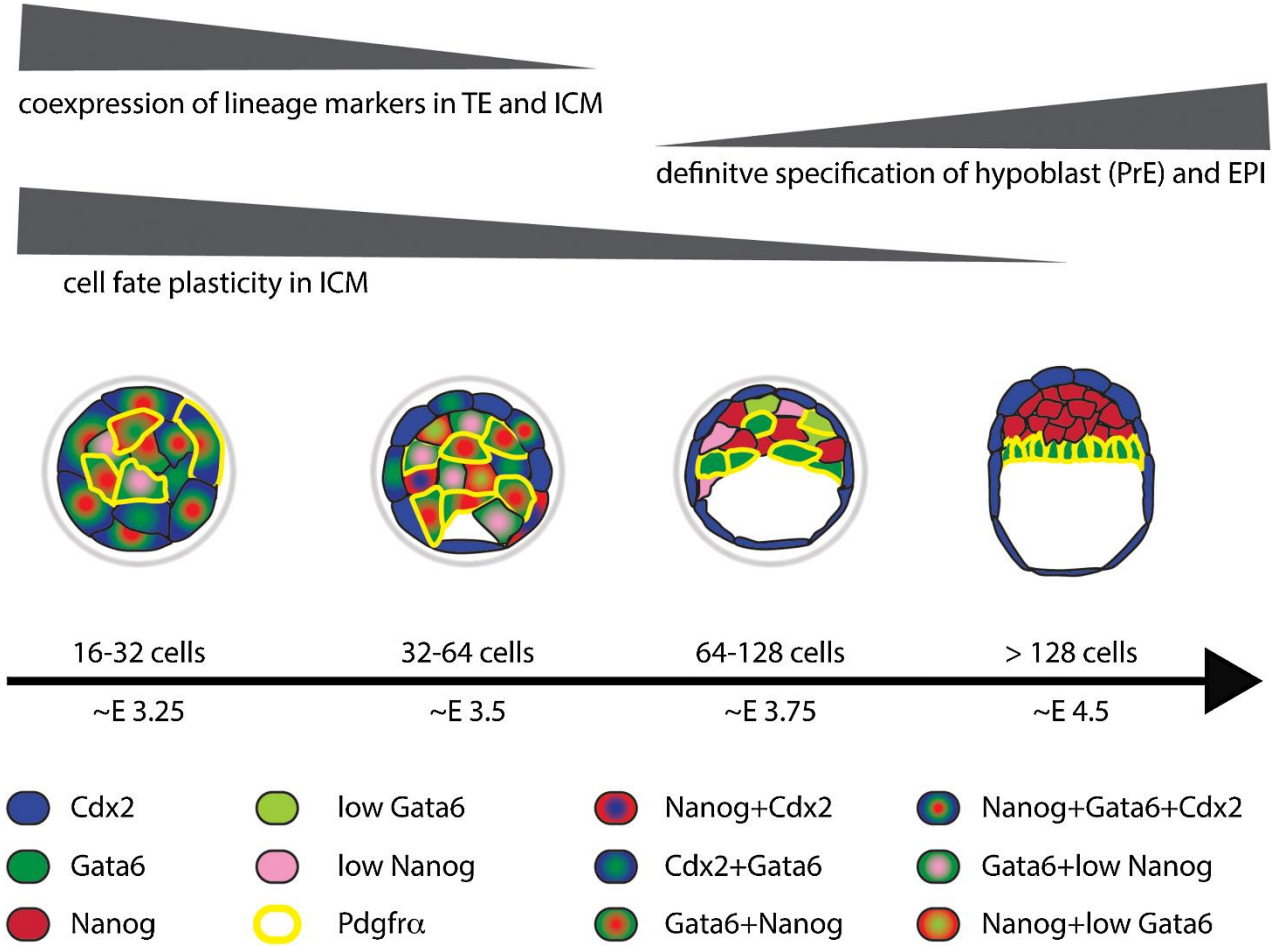

Figure 2: Model of gradual formation of embryonic lineages. The timeline indicates subsequent embryonic divisions and embryonic days (E). Initial stochastic and overlapping expression of lineage-specific markers is followed by a gradual switch towards an exclusive expression of these transcription factors (TFs). The first lineage to emerge is TE, with exclusive expression of CDX2 and other markers. Cells in the ICM express exclusively PrE (like GATA 6/4 and PDGFRa) and EPI markers (like NANOG), but initially they are distributed within ICM in salt and pepper fashion. Stabilized expression of lineage TFs contributes to the sorting process, initiated at ~64 cell stage. Sorting progression is governed by various cell behaviours and selective apoptosis. Plasticity of ICM cells gradually decreases, but they retain a certain degree of capability to change their fate up until the definitive specification and allocation of PrE and EPI, in embryos with more than ~128 cells. Colours represent the expression of lineage-specific TFs.

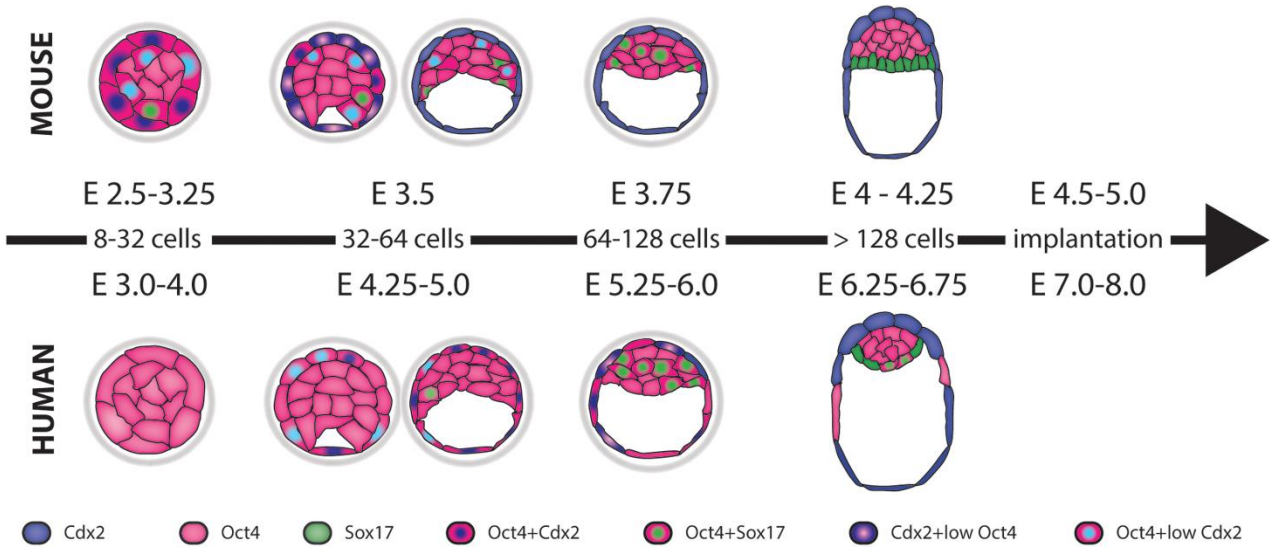

Figure 3: Developmental landmarks of human and mouse embryos. Human and mouse embryos share similar morphological characteristics, although the timing of events and expression pattern of lineage specific markers differs significantly between these two species. The timeline indicates subsequent divisions (reflected in number of cells in embryo). Embryonic days (E) are shown above and below the timeline. Colours represent the expression of lineage-specific TFs.

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https://doi.org/10.1093/molehr/gaw002